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An Eukaryotic RuvB-like Protein (RUVBL1) Essential for Growth*

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A human protein (RUVBL1), consisting of 456 amino acids (50 kDa) and highly homologous to RuvB, was identified by using the 14-kDa subunit of replication protein A (hsRPA3) as bait in a yeast two-hybrid system. RuvB is a bacterial protein involved in genetic recombination that bears structural similarity to subunits of the RF-C clamp loader family of proteins. Fluorescence *in situ* hybridization analysis demonstrated that the RUVBL1 gene is located at 3q21, a region with frequent rearrangements in different types of leukemia and solid tumors. RUVBL1 co-immunoprecipitated with at least three other unidentified cellular proteins and was detected in the RNA polymerase II holoenzyme complex purified over multiple chromatographic steps. In addition, two yeast homologs, scRUVBL1 and scRUVBL2 with 70 and 42% identity to RUVBL1, respectively, were revealed by screening the complete *Saccharomyces cerevisiae* genome sequence. Yeast with a null mutation in scRUVBL1 was nonviable. Thus RUVBL1 is an eukaryotic member of the RuvB/clamp loader family of structurally related proteins from bacteria and eukaryotes that is essential for viability of yeast.

Genetic recombination plays a critical role in maintaining gene diversification through chromosomal rearrangement and also genome stability through the repair of DNA damage. The activities of many proteins are required for recombination. In bacteria, for instance, RecA protein with the assistance of single-stranded DNA-binding protein promotes strand exchange with a homologous duplex and creates a four-strand intermediate or Holliday junction. The latter is then translocated by RuvA and RuvB proteins through branch migration and resolved by RuvC protein to yield recombinant DNA products (1). RuvB protein is a DNA-dependent ATPase and helicase that forms hexameric rings and has a low intrinsic affinity for DNA. RuvA is a structure-specific DNA-binding protein that has a high affinity for Holliday junctions and interacts with RuvB to form specific complexes with Holliday junctions. The presence of RuvA facilitates RuvB-mediated ATP hydrolysis and branch migration (2, 3).

Recombination activity has also been identified in eukaryotes and may be related to cell cycle progression. The Holliday intermediates in yeast accumulate to the highest level and become detectable during S phase (4). A yeast homolog of bacterial RecA, Rad51, is essential for spore formation during meiosis (5). Rad51 mRNA is significantly increased during meiosis and is also regulated during the mitotic cell cycle, with the highest levels found at the G_1/S boundary (6, 7). Homologs of bacterial RecA are also found in other eukaryotes, including *Xenopus laevis*, *Lilium longiflorum*, *Neurospora crassa*, *Arabidopsis thaliana*, mouse, chicken, and man (8), suggesting that the machinery involved in recombination is highly conserved among all organisms from bacteria to man. Consistently, single-stranded DNA-binding protein is also functionally conserved through evolution. Human single-stranded DNA-binding protein, also known as human replication protein A (hsRPA),[1] is a heterotrimer of 70, 32, and 14 kDa subunits. In both man and yeast, RPA serves as an important accessory factor in pairing and strand exchange carried out by Rad51 (9, 10).

Recent evidence suggests that recombination proteins may be physically associated with proteins involved in transcription. Tumor suppressor p53, a transcriptional activator for many important genes, has been demonstrated to interact with hsRPA (11) and with hRad51 to inhibit the activities of hRad51 in recombination (12). Tumor suppressor BRCA1 co-localizes at nuclear foci with hRad51 during S phase and co-immunoprecipitates with the same (13). However, BRCA1 is also a component of the RNA polymerase II holoenzyme (14) and has been implicated in transcriptional activation (15). Thus proteins like Rad51 and RPA, likely involved in recombination, physically interact with proteins involved in transcription.

In this paper, we report the identification of a human protein (RUVBL1) related in sequence to bacterial RuvB by using the 14-kDa subunit of human RPA (hsRPA3) as bait in a yeast two-hybrid system. The RUVBL1 gene is mapped to 3q21, a region with frequent rearrangements in different types of leukemia and solid tumors (16, 17). About 30% of the total cellular RUVBL1 co-purifies with the RNA polymerase II holoenzyme over multiple chromatographic steps. In addition, two yeast homologs scRUVBL1 (GenBank™ accession number S52968) and scRUVBL2 (GenBank™ accession number S61029) with 70 and 42% identity to RUVBL1, respectively, are revealed by screening the complete *Saccharomyces cerevisiae* genome sequence. Knockout of scRUVBL1 demonstrates that scRUVBL1 is essential for growth. Thus, RUVBL1 is an essential protein (in yeast) and is partly present in the RNA polymerase II holoenzyme complex.

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[1] The abbreviations used are: RPA, replication protein A; hs, *Homo sapiens*; pol II, RNA polymerase II; kb, kilobase pair(s); DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; CBP, CREB-binding protein; bp, base pair(s).

EXPERIMENTAL PROCEDURES

Cloning and Sequencing—pAS-hsRPA3 was constructed by transferring the *EcoRI-XhoI* fragment of pEGRPA3 (18) to pAS2 (CLONTECH). hsRPA3 was expressed as a fusion protein containing a Gal4 DNA-binding domain in yeast Y190 (19). A human lymphocyte MATCHMAKER cDNA library (CLONTECH) was used for yeast two-hybrid interaction with hsRPA3. The transformation and selection procedures were performed according to the CLONTECH manual with slight modifications. The library plasmids harboring RUVBL1 cDNA were extracted from the screened yeast and sequenced. RUVBL1 cDNA sequence has been deposited in the GenBank™ (accession number AF070735).

Fluorescence in Situ Hybridization—An ~4.8-kb human genomic clone containing RUVBL1 was identified by screening a human placenta genomic library (CLONTECH) using the RUVBL1 cDNA as probe. The genomic clone was labeled with digoxigenin-11-dUTP as described (20). Hybridization of metaphase chromosome preparations from peripheral blood lymphocytes obtained from normal human males was performed with the RUVBL1 gene at 15 μg/ml in Hybrisol VI according to a previously described method (21).

Cell Culture—Human WI38 fibroblasts, 293T transformed embryonic kidney cells, or HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Anti-RUVBL1 Antibody, Immunoprecipitation, and Immunoblotting—Anti-RUVBL1 antiserum was raised in a rabbit using a recombinant His_6-tagged fragment of RUVBL1 containing amino acids 61–456 created by cloning the fragment of RUVBL1 cDNA into the *XhoI* site of pRSETC (Invitrogen). The antibody was further immunopurified from the antiserum using purified RUVBL1 as antigen. Immunoblotting was performed according to standard protocols.

293T cells were labeled with [^{35}S]methionine for 6 h in methionine-free Dulbecco's modified Eagle's medium following a 4-h starvation and lysed in RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 0.1 mM PMSF). The lysate was precleared with preimmune serum bound to protein A-Sepharose for 1 h followed by a 1-h incubation with anti-RUVBL1 antiserum in the above lysis buffer. The precipitated complex was loaded on a 12% SDS-PAGE gel, and detected by autoradiography. To ensure that co-immunoprecipitating proteins were not a result of cross-reacting antibody, interactions were disrupted by lysing cells in 1% SDS at 100 °C. Samples were then diluted to RIPA buffer conditions and immunoprecipitated with the same antibodies.

Northern Blot (RNA) Analysis—Total RNA was extracted from HeLa cells as described (22). 10 μg of RNA/lane was separated on a formaldehyde-agarose gel and blotted to a nylon membrane. The blot was hybridized at 42 °C with a fragment of RUVBL1 cDNA encoding amino acids 61–456. The membranes were also hybridized with a 1.3-kb *HindIII-PstI* cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cDNA fragments of 1.7 and 1.4 kb containing the entire open reading frames of cyclins B and E, excised out of RcCyclin B and RcCyclin E plasmids with *HindIII* and *XbaI* (23).

Purification of RUVBL1 Expressed in Insect Cells—Full-length RUVBL1 coding sequence was cloned into *BamHI/XhoI* sites of pFastBac 1 (Life Technologies, Inc.) and transposed into a bacmid following the transformation of DH10 Bac (Life Technologies, Inc.). Then baculovirus bearing RUVBL1 was harvested from Sf9 insect cells transfected with the bacmid and employed to infect High 5 insect cells in Grace's insect medium supplemented with 10% heat-inactivated fetal bovine serum. The infected High 5 cells were harvested and lysed in lysis buffer (50 mM Tris acetate, pH 8.0, 150 mM KOAc, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5% Nonidet P-40, and 0.1 mM PMSF). The lysate was first passed through phosphocellulose column equilibrated with TEGD buffer (20 mM Tris acetate, pH 7.7, 1 mM EDTA, 10% glycerol, 1 mM DTT, and 1 mM PMSF). The flow-through (fraction 1) was directly loaded onto a Q Sepharose column equilibrated with 50 mM KOAc in TEGD buffer and eluted with a 50–500 mM KOAc gradient in TEGD buffer. RUVBL1 was in the 200–350 mM KOAc fraction. Following overnight dialysis in TEGD buffer plus 50 mM KOAc, the above fraction containing RUVBL1 (fraction 2) was loaded onto a Mono Q fast protein liquid chromatography column equilibrated with 50 mM KOAc in TEGD buffer and eluted with a 50–350 mM KOAc gradient in TEGD buffer. RUVBL1 was eluted with ~260 mM KOAc and precipitated with 50% saturated ammonium sulfate for 1 h. The precipitate was dissolved in RUVBL1 storage buffer (20 mM Tris acetate, pH 7.7, 50 mM KOAc, 10% glycerol, 0.02 mM EDTA, 1 mM DTT, and 1 mM PMSF), dialyzed in the same buffer at 4 °C, and stored at −70 °C. RUVBL1 was followed in the above different steps by SDS-PAGE of fractions and Western blot with αRUVBL1 antibodies. The RUVBL1 protein purified over these steps was at least 95% pure.

ATPase Assays—Two assays, a TLC assay and a coupled spectrophotometric assay, have been used to measure ATPase activity of bacterial RuvB. They are suitable for measuring ATP hydrolysis rates at ATP concentrations below and above 125 μM, respectively. In the TLC assay (24, 25), reactions were carried out at 37 °C in the absence or presence of various DNAs including single-stranded or double-stranded linear DNAs, circular plasmid or phage DNAs, and synthetic Holliday junction DNAs at 20–200 μM (nucleotides). The reaction mixtures contained 20 mM Tris-HCl at pH 6.8–8.0, 1–32 mM $MgCl_2$, 1 mM DTT, 100 μg/ml bovine serum albumin, 25–1300 μM ATP, 40 μCi/ml [α-^{32}P]ATP, 0–0.5 μM hsRPA (18), and 0.6–4.0 μM purified RUVBL1. The reactions were stopped by addition of EDTA to 40 mM. Aliquots (1 μl) of reaction were spotted at various time points (1–60 min) onto polyethyleneimine-cellulose TLC plates, which were developed in 1 M formic acid/0.5 M LiCl. Hydrolysis of [α-^{32}P]ATP into [α-^{32}P]ADP was determined by autoradiography.

The coupled spectrophotometric assay in which ATP hydrolysis was coupled with oxidation of NADH (24) employed pyruvate kinase and lactate dehydrogenase as an ATP regeneration system in addition to the reaction components supplemented in TLC assay. Because the oxidation of NADH can be detected at 380 nm by spectrophotometer, [α-^{32}P]ATP was omitted from the reaction mixture. An NADH extinction coefficient of $\epsilon_{380} = 1.21\ mM^{-1}\ cm^{-1}$ was used to calculate the rate of ATP hydrolysis.

Branch Migration and DNA Helicase Assays—Two assays using synthetic Holliday junctions and primer/template duplexes as substrates, respectively, were employed to measure branch migration and helicase activity of RUVBL1. Synthetic Holliday junctions were prepared essentially as described previously (26). The asymmetric Holliday junction was constructed from four oligonucleotides (oligos 1–4) with 88 or 89 bases. Oligo-1 was 5′-^{32}P-labeled prior to annealing using T4 polynucleotide kinase and [γ-^{32}P]ATP. Annealed junctions were purified by gel electrophoresis. The partial duplex markers used in the experiment shown in Fig. 4*B* were prepared by annealing 200 ng of ^{32}P-labeled oligo-1 with excess oligo-2 or -4. To determine the activity of RUVBL1, the reaction mixture (20 μl) contained ~2.5 ng of ^{32}P-labeled synthetic Holliday junction DNA in 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 1 mM ATP, and 0.6–4.0 μM RUVBL1 (26). Reactions lasted for 15–60 min at 37 °C and were stopped and deproteinized by the addition of 2 μl of 10× stop buffer to a final concentration of 20 mM Tris-HCl, pH 7.5, 25 mM EDTA, 0.5% SDS, and 2 mg ml^{-1} proteinase K. The samples were analyzed using a 6% polyacrylamide gel with a Tris borate buffer system. ^{32}P-Labeled DNAs were detected by autoradiography.

For simple helicase assays two primer/template duplexes were prepared in the same manner as the synthetic Holliday junctions. Two template oligonucleotides, 5′ to 3′ template and 3′ to 5′ template were used with their 3′ and 5′ ends, respectively, annealed to the ^{32}P-labeled primer. The DNA sequences for these oligonucleotides are the followings: primer, 5′-TGGTATCGTGAGCACTGCAGCCAGGATCAT-3′; 5′ to 3′ template, 5′-TCTCCCTATAGTGAGTCGTATTTTTGATCC-TGGCTGCAGTGCTCACCATACCA-3′; 3′ to 5′ template, 5′-ATGATC-CTGGCTGCAGTGCTCACCTTACCTTCTCCCTATACTGAGTCGTAT-TT-3′. Reaction mixtures contained 10 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 2 mM dATP or GTP or dNTP or NTP, 400 μg/ml bovine serum albumin, 2.5 nM of the annealed duplexes, and 1 μM RUVBL1. Following 2 h of incubation at 30 °C, the reactions were stopped by addition of stop buffer to a final concentration of 0.33% SDS, 17 mM EDTA, 14% glycerol, and 0.01% bromphenol blue. The samples were analyzed on an 18% polyacrylamide gel with a Tris borate buffer system. ^{32}P-Labeled DNAs were detected by autoradiography.

Purification of RNA Polymerase II Holoenzyme—pol II holoenzyme was purified from HeLa whole cell extracts through Bio-Rex 70 column, sucrose step gradient, and nickel nitrilotriacetate as described previously (14, 27). Pull-down of RNA polymerase holoenzyme from cell extracts by GST-CBP (containing amino acid residues 1805–1890 of CBP fused to glutathione *S*-transferase) or GST-BRCA1 (containing amino acid residues 1560–1863 of the familial breast cancer susceptibility gene product, BRCA1) has also been described in detail (27, 28).

scRUVBL1 Knockout and Rescue in Yeast—scRUVBL1 was amplified from yeast genomic DNA using polymerase chain reaction primer 1 (5′-CATGCCATGGTCGCTATCAGTGAAGTCA-3′; the ATG corresponding to the initiator methionine of scRUVBL1, GenBank™ accession number S52968) and primer 2 (5′-GGGGGATCCTTACAAA-TAATTTGCGGAAGTT-3′; the TTA is antisense to the termination codon TAA of the scRUVBL1 sequence). The 1.4-kb product containing

the entire open reading frame of scRUVBL1 was blunted at the *Nco*I site (by polymerase fill-in reaction) and inserted into pKS$^+$ (Stratagene) between the *Eco*RV and *Bam*HI sites in the polylinker. To knockout scRUVBL1, pKS$^+$-scRUVBL1 was digested with *Bgl*II and *Eco*RV, removing a 420-bp internal segment of the scRUVBL1 gene. This internal segment was replaced by a 1.2-kb *Hin*dIII fragment carrying the URA3 gene such that it was flanked by 630 bp of the 5′ and 330 bp of the 3′ end of scRUVBL1. The entire scRUVBL1-URA3 cassette was removed from the plasmid with *Sal*I and *Bam*HI and transformed into the diploid *S. cerevisiae* strain YSB455 (MATa/MATα ura3–52/ura3–52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 lys2Δ202/lys2Δ202). Several URA$^+$ transformants were selected. Restriction digest and Southern analysis of genomic DNA identified several colonies with successful deletion of one copy of the scRUVBL1 gene. Sporulation and tetrad dissection was conducted for two independently derived yeast strains with a deletion of scRUVBL1 according to standard protocols.

To rescue the above yeast strains bearing a heterozygous deletion of scRUVBL1, a cDNA fragment containing scRUVBL1 open reading frame was inserted into an ectopic expression vector Yep51 (18) between *Sal*I and *Bam*HI sites. Following transformation of the strains with Yep51-scRUVBL1, the tetrads were sporulated and dissected. The strains were also transformed with Yep51 or Yep51-RUVBL1 instead of Yep51-scRUVBL1. To confirm the results of dissection, each haploid was tested by patch-mating with tester strains MAT *a met* and MAT α *met*, respectively.

RESULTS

Cloning of RUVBL1 Using Yeast Two-hybrid System—The yeast two-hybrid system is a sensitive *in vivo* method for identifying genes encoding proteins that interact with a protein of interest. Using hsRPA3 (the 14-kDa subunit of hsRPA) as a bait in the yeast two-hybrid system to screen a human cDNA library, we identified several cDNAs that encode potential hsRPA3-interacting proteins including one for RPA1 (the 70-kDa subunit of hsRPA) and a 1.8-kb novel cDNA. The latter encodes a protein of 456 amino acids (50 kDa), referred to as RUVBL1, and is a human homolog of the recently identified rat TBP-interacting protein (TIP49) (29). Amino acid sequences of RUVBL1 and TIP49 proteins are identical except that Ile^{291} in RUVBL1 is replaced by Val in TIP49. As reported previously (29), TIP49 shares high homology with RuvB proteins from different bacteria including *Thermus aquaticus thermophilus* (Ref. 30; GenBank™ accession number U38840), *Thermotoga maritima* (30), *Mycobacterium leprae* (GenBank™ accession number U00011) and *Borrelia burgdorferi* (GenBank™ accession number Y08885). As shown in Fig. 1, two regions of RUVBL1 (amino acids 26–88 and amino acids 277–425) are homologous to the RuvB sequence of *T. thermophilus* (30). *T. thermophilus* RuvB consists of 324 amino acids, and its amino acids 1–226 were aligned with the two regions of RUVBL1 in Fig. 1 (*A* and *B*). The two homologous regions between the two proteins are 25 and 38% identical, respectively, and 46 and 54% similar, respectively. The regions of homology contain Walker A and B motifs but are not restricted to just those motifs. Walker A (Gx4GKT) and B (4 hydrophobic-DExH/N) motifs are involved in ATP binding and/or ATP hydrolysis of DNA/RNA helicases (31, 32). RUVBL1 contains an insertion of approximately 190 amino acids between the two regions. In a recent paper the bacterial RuvB protein was suggested to be structurally similar to subunits of RF-C and other clamp loader protein complexes associated with replicative DNA polymerases from multiple species (33). In support of this, we note a moderate sequence homology between RUVBL1, RuvB, and DNA polymerase III γ and τ subunits (GenBank™ accession number g580914) of *Bacillus subtilis* (Fig. 1*B*). Interestingly, like RUVBL1, the subunits of clamp loader protein complexes have insertions of different sizes between the regions containing the Walker A and B motifs (Ref. 33 and Fig. 1*B*). Taken together, we suggest that RUVBL1 is structurally a part of the RuvB/clamp loader subunit family of proteins.

Two putative yeast genes were identified in the *S. cerevisiae* genome encoding proteins scRUVBL1 and scRUVBL2 with 70 and 42% identity to RUVBL1, respectively. They are highly homologous to RUVBL1 over both the regions containing the Walker A and B motifs and over the third inserted region between the two motifs (Fig. 1, *A* and *B*). Because protein complexes involved in DNA repair and replication are remarkably well conserved in sequence and subunit composition between mammals and yeast, we anticipate that there are likely to be at least two related RUVBL proteins in humans. The human gene identified is more similar to scRUVBL1 than scRUVBL2 leading us to tentatively identify it as RUVBL1.

The human RUVBL1 gene was mapped to chromosome 3 in band q21 (3q21) using fluorescence *in situ* hybridization. Map position was determined by visual inspection of the fluorescent hybridization signals on 4,6-diamidino-2-phenylindole-dihydrochloride-stained metaphase chromosomes. In 18 of 20 metaphase preparations analyzed, hybridization signal was found to be present on the long arm of chromosome 3 in band q21; in 12 metaphase spreads both copies of chromosome 3 were labeled; and in 6 metaphase spreads signal was detected on one chromosome 3.

RUVBL1 mRNA Expression Is Constant through the Cell Cycle—Because Rad51 mRNA is regulated during the cell cycle (6, 7), it was interesting to determine whether the expression of RUVBL1 is similarly regulated. RUVBL1 mRNA levels were examined by Northern blot analysis of RNA from synchronous HeLa cells released from an M phase block by nocodazole (Fig. 2). The progressive decrease in cyclin B and increase in cyclin E mRNA levels indicates that the cells passed through G_1 and S synchronously. However, RUVBL1 mRNA was detected at a constant level during the cell cycle in comparison with the GAPDH control. Thus, unlike Rad51, which shows increased expression in S phase (6, 7), RUVBL1 mRNA is not cell cycle-regulated.

A 50-kDa RUVBL1 Protein Is Detected in Human Cell Lines—Antiserum against RUVBL1 was raised from a rabbit immunized with the His-tagged fragment of RUVBL1 (amino acids 61–456) overexpressed in *Escherichia coli*. Western blot with this antiserum detected a 50-kDa protein of the expected size in the following human cell lines: 293T (embryonic kidney cells transformed with adenovirus and SV40 T antigen), MCF7 (breast cancer cells), HeLa (cervical cancer cells), and WI38 (primary fibroblasts) (Fig. 3*A*, *lanes 1* and *2* and data not shown).

Because bacterial RuvB alone promotes branch migration and hydrolyzes ATP, we asked if the same was true for RUVBL1. RUVBL1 protein was overexpressed in High 5 insect cells infected with a recombinant baculovirus. In comparison with uninfected cells, a protein of 50 kDa matching the predicted size of intact RUVBL1 and three smaller breakdown products were detected in the infected cells by immunoblotting with anti-RUVBL1 antibody (Fig. 3*A*, *lanes 3–6*). Intact RUVBL1 was purified by conventional chromatography to >95% purity as shown in Fig. 3*B* (*lane 2*).

Unexpectedly, the purified RUVBL1 expressed in the insect baculovirus system did not hydrolyze ATP. Fig. 4*A* shows that RUVBL1 did not have any ATPase activity detected with TLC assay in the presence of 25 μM ATP. The results were still negative in the presence of higher concentrations of ATP up to 1.3 mM using both the TLC and the coupled spectrophotometric assays under the different buffer conditions described under "Experimental Procedures" despite the addition of different types of DNA substrates. There was no eukaryotic RUVB protein to be used as a positive control, and the conditions required for a prokaryotic RUVB protein may well be different from

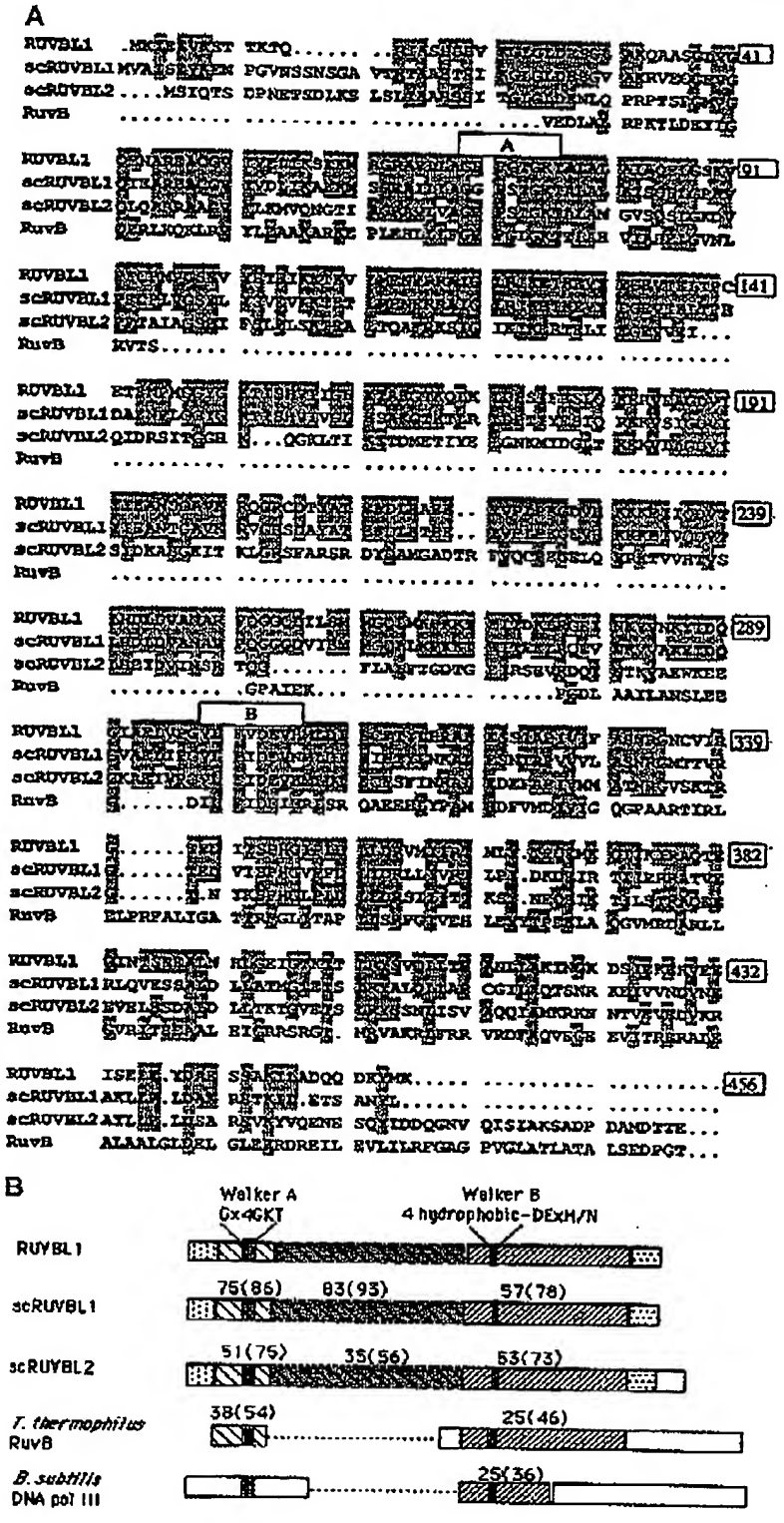

FIG. 1. *A*, the full-length sequence of RUVBL1, aligned by GCG Pileup program to show the identities with full-length scRUVBL1 (YDR190C on chromosome IV from coordinates 842032 to 840641) and scRUVBL2 (YPL235W on chromosome XVI from coordinates 103232 to 104647) as well as amino acids 1–280 of eubacteria *T. thermophilus* RuvB. The *shaded boxes* indicate the matches between RUVBL1 and any other sequences. The *dots* indicate gaps introduced in the sequences for optimal alignment. The sequences marked A and B are the Walker A and B motifs. *B*, a schematic representation of the eukaryotic RUVBL proteins, bacterial *T. thermophilus* RuvB protein and one example of a bacterial DNA polymerase clamp loader protein (*B. subtilis* DNA polymerase III; Ref. 42). The percentages of identity and similarity (in parenthesis) between each of the regions and the corresponding *shaded regions* of RUVBL1 are indicated.

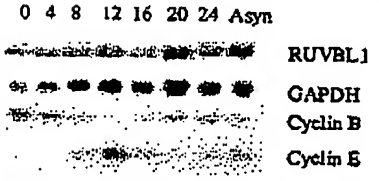

FIG. 2. **Expression of RUVBL1 mRNA through the cell cycle.** Levels of RUVBL1 mRNA in HeLa cells were examined by Northern blot analysis following the indicated hours of release *from* nocodazole block. The levels of cyclin B (expressed in G_2/M) and cyclin E (G_1) are shown as a reference of the cell cycle stage. GAPDH is a reference of the amount of RNA loaded. The 20-h sample is overloaded (see GAPDH control).

those that are optimal for the eukaryotic RUVBL1. Thus, High 5 cell lysate was utilized as a positive control to determine the sensitivity of the assays in detection of ATP hydrolysis (*lane 2* in Fig. 4*A*, and data not shown).

In the case of bacterial RuvBs, ATPase activity is required for helicase or branch migration activities. Therefore, it was unlikely that the purified RUVBL1 should possess any helicase activity. Indeed, the purified RUVBL1 failed to cause branch migration in a synthetic Holliday junction substrate (Fig. 4*B*). The RUVBL1 protein also failed to displace the primer strand on the primer-template duplexes described under "Experimental Procedures," suggesting that it did not have 5′-3′ or 3′-5′ helicase activities (data not shown).

RUVBL1-associated Cellular Proteins—We asked whether RUVBL1 was associated with any other cellular proteins. Immunoprecipitation of human cell lysate showed that RUVBL1 was associated with at least three other unidentified proteins. 293T cells were metabolically labeled with [^{35}S]methionine, and cellular proteins immunoprecipitated with anti-RUVBL1 antiserum. At least four bands of 160, 70, 50, and 45 kDa, respectively, were identified following immunoprecipitation (Fig. 5, *lane B*). To determine which band was from the direct interaction with anti-RUVBL1 antibodies, the cell lysate was prepared in parallel by lysing cells in 1% SDS and denatured by heating at 100 °C for 10 min. As shown in *lane D* of Fig. 5, only a 50-kDa band was detected from the denatured lysate, indicating that the 50-kDa peptide itself is RUVBL1, and the other three are RUVBL1-associated polypeptides.

Although RUVBL1 interacted with hsRPA in the yeast two-hybrid system, none of the RUVBL1 co-precipitated proteins were recognized by antibodies against hsRPA1, hsRPA2, or hsRPA3. Therefore, we have not been able to detect a stable complex between RUVBL1 and hsRPA. No ATPase or helicase activity was detected from the immunoprecipitates.

RUVBL1 Copurifies with RNA Polymerase II Holoenzyme Complex—It is possible that RUVBL1 is present in a larger complex of proteins and that the immunoprecipitation conditions disrupted such a complex. Gentler chromatographic conditions were employed to examine this issue. HeLa whole cell extract was applied to a Bio-Rex 70 column and eluted with increasing concentrations of potassium acetate. RUVBL1 was found in all fractions, although most was detected in the flow-through and the 0.6 M potassium acetate elute; the latter also contained about a half of pol II (Fig. 6*A*).

Because the 0.6 M Bio-Rex 70 fraction contains RNA pol II, we tested whether RUVBL1 was in the RNA polymerase holoenzyme complex. The 0.6 M Bio-Rex 70 column protein fraction was centrifuged through a sucrose gradient and analyzed by Western blotting (Fig. 6*B*). RUVBL1 and pol II appeared in a major peak centering on fractions 13–19. The holoenzyme-specific polypeptides cyclin C and cdk 8 are associated in these fractions (27). Thus, RUVBL1 co-eluted with the RNA pol II holoenzyme.

Holoenzyme peak fractions from the sucrose gradient were then subjected to metal chelate chromatography, and RUVBL1 again co-eluted with pol II (Fig. 6*C*). About 50% of RUVBL1 bound to the metal chelate matrix and co-eluted with pol II in fractions 11–13. As determined before (14), following the metal chelate chromatography, the holoenzyme was purified about 400-fold. About 30% of the total cellular RUVBL1 co-purified with the pol II holoenzyme.

Affinity matrices containing either residues 1805–1890 of CBP or residues 1560–1863 of BRCA1 have been demonstrated to specifically bind the pol II holoenzyme (27). As shown in Fig. 6*D*, RUVBL1 was also detected in the complex pulled down by either CBP or BRCA1, further suggesting that RUVBL1 is present in the pol II holoenzyme.

scRUVBL1 Is Essential for Growth—To test the biological importance of RUVBL1, one copy of *scRUVBL1* was deleted in diploid yeast by homologous recombination (Fig. 7*A*). Diploid yeast were transformed with a 2.2-kb linear DNA fragment containing the URA3 gene partially replacing scRUVBL1 sequence in open reading frame (Fig. 7*A*, *panel b*). Genomic DNA from transformed diploids was digested with *Bgl*II and probed on a Southern blot with a 630-bp fragment of *scRUVBL1*. Homologous recombination at the *scRUVBL1* locus removes a *Bgl*II site in the gene so that the probe should detect a fragment of 4230 bp instead of a fragment of 1770 bp from the wild type gene. In several of the diploids two bands of 4.2 and 1.8 kb were detected, indicating that the *URA3* gene was successfully integrated in one allele of *scRUVBL1* (Fig. 7*A*, *panel c*). Sporulation of these diploids and subsequent dissection of 12 tetrads resulted in a segregation of 2:2 for viability following meiosis (Fig. 7*B*, *panel 1*). All viable spores consistently lack URA3, which marked the deleted scRUVBL1 allele. Microscopic examination of the spores that failed to grow up into visible colonies revealed only four or five large budded cells from each spore, suggesting that scRUVBL1$^-$ yeast are nonviable. To further confirm this result, the yeast strain with heterozygous deletion of scRUVBL1 was transformed with a vector expressing ectopic scRUVBL1. 10 of 20 dissected tetrads grew into four viable colonies in the strains expressing ectopic scRUVBL1 (Fig. 7*B*, *panel 2*). Colonies from the dissected tetrads were verified as haploids by mating and segregated 2:2 for *URA3* markers. In contrast, the strains transformed with the one containing human RUVBL1 (Fig. 7*B*, *panel 3*) or the empty vector (Fig. 7*B*, *panel 4*) produced only two viable colonies for each of 20 tetrads. Thus, the defect in scRUVBL1$^-$ yeast was rescued by extragenic scRUVBL1 under the control of a heterologous promoter but not by human RUVBL1. These data indicate that scRUVBL1 is essential for viability of yeast *S. cerevisiae*.

DISCUSSION

In the studies reported here, we identified a human protein RUVBL1 that interacts with hsRPA3 in a yeast two-hybrid system and is structurally similar to bacterial RuvB. The RUVBL1 gene was mapped to 3q21, a region with frequent rearrangements in different types of leukemia and solid tumors (16, 17). Thus, the assignment of the RUVBL1 gene to 3q21 encourages us to investigate in the future whether this gene is close to the rearrangement breakpoint and whether the gene or its expression is altered in tumors with this cytogenetic anomaly. In addition, two yeast homologs scRUVBL1 and scRUVBL2, which are 70 and 42% identical to RUVBL1, respectively, were revealed by screening the complete *S. cerevisiae* genome. Furthermore, we showed that RUVBL1 is present in the RNA polymerase II holoenzyme and that its yeast homolog scRUVBL1 is essential for growth.

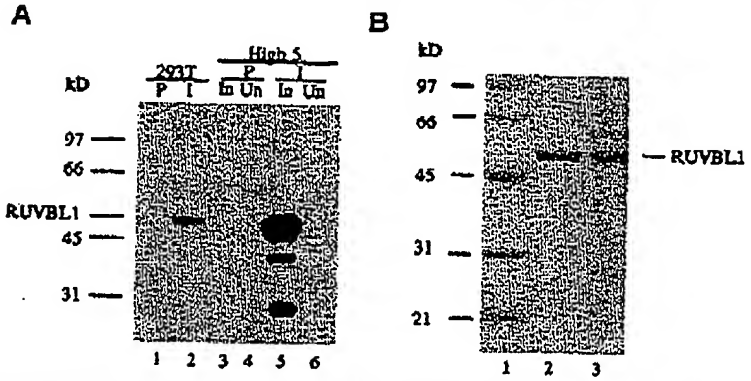

FIG. 3. **Identification and purification of RUVBL1.** *A*, identification of RUVBL1 by Western blot. S100 extract of human 293 cells and the lysate from High 5 insect cells infected (*In*) or uninfected (*Un*) with baculovirus bearing RUVBL1 cDNA were separated on 10% SDS-PAGE and blotted with rabbit preimmune serum (*P*) or immune antiserum against RUVBL1 (*I*). *B*, identification of purified RUVBL1 by SDS-PAGE and Coomassie Blue staining. *Lane 1*, protein marker; *lane 2*, purified RUVBL1; *lane 3*, the lysate of High 5 cells infected with baculovirus bearing RUVBL1 cDNA.

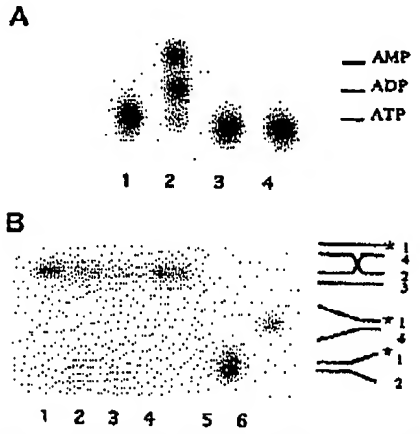

FIG. 4. **Purified RUVBL1 had no ATPase and helicase activities.** *A*, ATPases activity of RUVBL1 was tested with TLC in the reaction mixtures containing 20 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 1 mM DTT, 100 μg/ml bovine serum albumin, 25 μM ATP, 40 μCi/ml [α-^{32}P]ATP, 0.5 μM hsRPA, and either 0 μM (*lane 1*) or 1.2 μM of RUVBL1 (*lanes 3* and *4*) in the absence (*lane 1*) or presence of 100 μM (nucleotides) λ phage DNA (*lane 3*) or 20 μM (nucleotides) synthetic Holliday junctions (*lane 4*). *Lane 2* was a positive control in which RUVBL1 was replaced with High 5 cell lysate containing 0.5 mg/ml protein in the absence of DNAs. Reactions were carried out for 30 min at 37 °C, and the hydrolysis of ATP was assayed as described under "Experimental Procedures." *B*, branch migration assays were carried out using ^{32}P-labeled synthetic Holliday junctions as substrates in the reaction mixtures without (*lane 1*) or with the purified RUVBL1 at 1.2 μM (*lane 4*) or the RUVBL1-containing fraction 1 (*lane 2*; 0.24 mg/ml protein) or fraction 2 (*lane 3*; 0.23 mg/ml protein) as described under "Purification of RUVBL1 Expressed in Insect Cells" under "Experimental Procedures." After 30 min, the products were analyzed by gel electrophoresis and autoradiography. *Lanes 5* and *6* were the markers for partial duplexes, the products expected following complete branch migration.

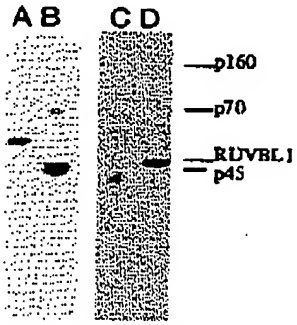

FIG. 5. **Determination of RUVBL1-associated proteins.** ^{35}S-Labeled proteins in undenatured (*lanes A* and *B*) or denatured (*lanes C* and *D*) 293T cell lysates were immunoprecipitated by preimmune serum (*lanes A* and *C*) or immune anti-RUVBL1 anti-serum (*lanes B* and *D*), separated on 12% SDS-PAGE gel, and detected by autoradiography. The polypeptides seen in the preimmune lanes were nonspecific and variable between different experiments.

Despite the sequence similarity between RUVBL1 and bacterial RuvB, we have not been able to detect ATPase and helicase activities using purified RUVBL1 expressed in baculovirus. This failure could result from inactivation of the protein during its purification from insect cells. Alternatively, the absence of these activities may reflect a requirement for additional reaction partners. One candidate partner is a RUVBL2 protein. It is interesting that biochemical studies of the bacterial RuvB hexamer have shown that only two of the six subunits bind ATP (34), indicative of a functional asymmetry in the RuvB hexamer. It is conceivable that a heteromer of RUVBL1 and RUVBL2 is necessary for ATP hydrolysis and strand displacement activity. Ongoing biochemical studies on yeast RUVBL proteins will test this hypothesis. As discussed earlier, the conservation of DNA replication and repair factors between yeast and humans make it likely that there is a human RUVBL2. Indeed, we have identified partial human cDNA sequences that appear to encode a related protein with more similarity to scRUVBL2 than to scRUVBL1. Whether these sequences truly represent human RUVBL2 will become evident once we have isolated full-length cDNA clones.

RUVBL may require partners unrelated to RuvB for its ATPase and helicase activities. Even though bacterial RuvB alone has weak ATPase and helicase activities *in vitro*, its *in vivo* activities require RuvA as a partner (35). A good candidate of a RuvA homolog is not obvious in the *S. cerevisiae* genome based on sequence analysis alone. However, this does not preclude the existence of such an eukaryotic RuvA-like molecule. As demonstrated in Fig. 5, RUVBL1 is associated with at least three other unidentified metabolically labeled proteins. Perhaps one of these RUVBL-associated proteins will emerge as an eukaryotic RuvA homolog, and our failure to observe ATPase or helicase activities in the immunoprecipitates may be caused by antibody inhibition or substoichiometric amounts of the part-

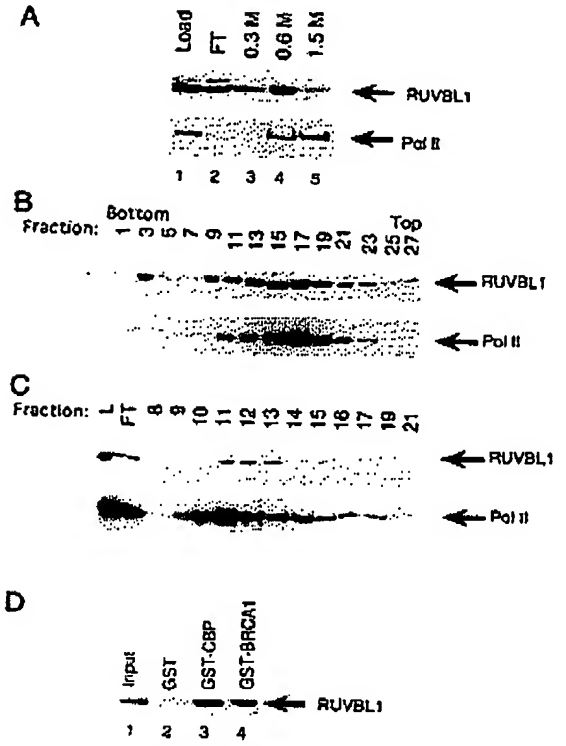

FIG. 6. **RUVBL1 copurified with RNA pol II holoenzyme.** *A*, HeLa whole cell extracts were chromatographed on Bio-Rex 70 matrix, and bound proteins were step-eluted at 0.3, 0.6, and 1.5 M KOAc. Protein samples from each fraction were subjected to SDS-PAGE, and blots were probed with RUVBL1 and pol II antibodies. *B*, the Bio-Rex 70 0.6 M fraction was subjected to centrifugation through a 10–60% sucrose gradient. After centrifugation, samples were collected and examined by Western blot with antibodies against RUVBL1 and pol II large subunit. *C*, fractions from sucrose sedimentation step were pooled and subjected to metal chelate chromatography. Fractions were eluted with a linear 5–130 mM gradient of imidazole. The indicated fractions were subjected to immunoblot analysis and probed with antibodies specific to RUVBL1 and pol II large subunit. *D*, GST fusion proteins GST-CBP (1805–1890) and GST-BRCA1 (1560–1863) were prebound to glutathione-agarose beads and then incubated with the fractions from Bio-Rex70 0.6 M elution step. Following washing in buffer containing 0.25 M KOAc and 0.5% Nonidet P-40, bound proteins were subjected to Western blotting and probed with immunopurified anti-RUVBL1 antibodies.

ner proteins. Additional proteins are also associated with RUVBL1 under gentler conditions of extraction (*e.g.* the polypeptides of the RNA polymerase II holoenzyme complex). ATPase and helicase activities have been reported in the RNA polymerase holoenzyme (36), and some of this may be due to RUVBL1 and its partners. Finally, RUVBL1 was shown to interact with hsRPA3 by the yeast two-hybrid assay but not by immunoprecipitation, indicating that low level or transient interactions of RUVBL1 with functional partners may be disrupted under conditions where the protein is extracted from cells. It may then be impossible to identify ATPase and helicase activity of RUVBL1 until the functional complex is reconstituted from recombinant proteins. Because we have a phenotype from the deletion of scRUVBL1, genetic complementation experiments in yeast will answer whether the Walker A and B motifs of scRUVBL1 are essential for the normal function of this protein. If the Walker A and B motifs prove essential genetically, biochemical experiments to demonstrate ATP binding (and hydrolysis) by scRUVBL1 (with or without putative partners) may be more successful.

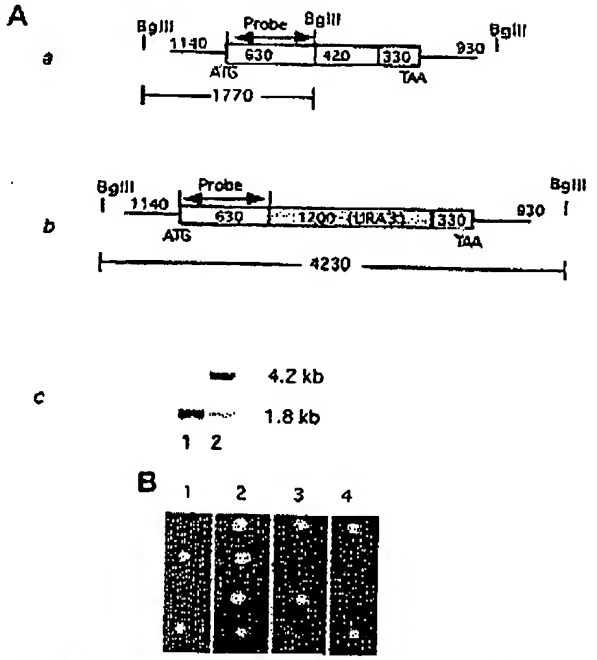

FIG. 7. **scRUVBL1 was essential for growth of yeast.** *A*, deletion of one allele of scRUVBL1. *Panels a* and *b* describe schematically wild type and partially replaced scRUVBL1 sequences, respectively. A deletion of one allele of *scRUVBL1* was detected by Southern blot of genomic DNA from scRUVBL1$^{+/}$ (*panel c*, *lane 1*) or scRUVBL1$^{+/-}$ (*panel c*, *lane 2*) diploids with indicated probe according to standard protocols. *B*, diploid yeast with heterozygous knockout at the scRUVBL1 locus were either sporulated and dissected directly (*panel 1*) or following transformation with Yep51-scRUVBL1 (*panel 2*), Yep51-RUVBL1 (*panel 3*), or Yep51 (*panel 4*).

BRCA1, which co-localizes and co-immunoprecipitates with hRad51 (13), is also a component of the RNA polymerase II holoenzyme (14), suggesting that DNA recombination repair proteins may be loosely associated with complexes involved in transcription. In this vein, it has also been noted that hRad51 and hsRPA, both of which are involved in DNA recombination repair, are present in RNA polymerase II holoenzyme under certain conditions (37). Recombination has been shown to be involved in the repair of mistakes ensuing from DNA replication and may be required for the completion of DNA replication. For example, mutations in DNA polymerases, ligases, topoisomerases, and DNA helicases all lead to increased mitotic recombination. The viability of some replication mutants also depends on recombination (4). These lines of evidence suggest that recombination plays a critical role in protecting genomic DNA from damage or mistakes. Genomic DNA in somatic cells is exposed to exogenous and endogenous damaging species throughout life. It is very important to have mechanisms to repair damaged DNA prior to transcription. Nucleotide excision repair has been linked to transcription. Proteins involved in nucleotide excision repair are found physically associated with transcription factors like TFIIH, facilitating the efficient repair of transcriptionally active areas of the genome (38–40). Based on its homology to RuvB, RUVBL1 is expected to be involved in recombination repair. As discussed above, BRCA1 is implicated in recombination repair because of its association with hRad51. Thus, the association of RUVBL1 and BRCA1 with the pol II holoenzyme complex may indicate that some aspects of recombination repair is similarly linked to transcription.

We did not expect a cellular component involved in recombination to be essential for viability. Although targeted disruption of the Rad51 gene leads to lethality in embryonic mice (41), a Rad51 null mutant of *S. cerevisiae* survives (5). Of course, other proteins in yeast may substitute for an essential function of Rad51. It is possible that recombination repair and scRUVBL1 are essential for accurate replication of the genome. The absence of scRUVBL1 may cause incomplete replication and result in cell death. Alternatively, if the co-purification of RUVBL1 with RNA polymerase holoenzyme implies that RUVBL1 has an essential function in transcription, the nonviability of scRUVBL1$^-$ yeast could be due to a global failure in transcription. Finally, there are other helicases in the RNA polymerase holoenzyme, including subunits of TFIIH and RNA helicase A, that facilitate the activation of transcription of specific promoters (35, 38). Thus, RUVBL1 may be essential for activation of transcription of certain essential genes. In conclusion, the sequence homology and biochemical fractionation data on RUVBL1 suggest that it is involved in recombination repair and/or transcription. Whether or not these or other unknown activities of RuvB make scRUVBL1 essential for viability remains to be determined.

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